

**UNITED STATES AIR FORCE
RESEARCH LABORATORY**

**TOXICITY OF EXPERIMENTAL JET
FUEL SYSTEM ICE-INHIBITING
AGENTS: II. GENE EXPRESSION
RESPONSE PROFILING**

K.T. Geiss

MANTECH-GEO-CENTERS JOINT VENTURE
P.O. BOX 31009
DAYTON, OH 45437-0009

J.M. Frazier

OPERATIONAL TOXICOLOGY BRANCH, AFRL/HEST
2856 G STREET
WRIGHT PATTERSON AFB, OH 45433-7400

May 1999

Interim Report - July 1997 - April 1999

20060630289

**Human Effectiveness Directorate
Deployment and Sustainment Division
Operational Toxicology Branch
2856 G Street
Wright-Patterson AFB OH 45433-7400**

Approved for public release; distribution is unlimited.

STINFO COPY

NOTICES

When US Government drawings, specifications or other data are used for any purpose other than a definitely related Government procurement operation, the Government thereby incurs no responsibility nor any obligation whatsoever, and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise, as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

Please do not request copies of this report from the Air Force Research Laboratory. Additional copies may be purchased from:

National Technical Information Service
5285 Port Royal Road
Springfield, Virginia 22161

Federal Government agencies and their contractors registered with the Defense Technical Information Center should direct requests for copies of this report to:

Defense Technical Information Service
8725 John J. Kingman Rd., Ste 0944
Ft. Belvoir, Virginia 22060-6218

DISCLAIMER

This Technical Report is published as received and has not been edited by the Technical Editing Staff of the Air Force Research Laboratory.

TECHNICAL REVIEW AND APPROVAL

AFRL-HE-WP-TR-2000-0109

This technical report has been reviewed and is approved for publication.

FOR THE DIRECTOR



DAVID R. MATTIE, Ph.D.
Acting Branch Chief, Operational Toxicology Branch
Air Force Research Laboratory

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE May 1999		3. REPORT TYPE AND DATES COVERED Interim Report - July 1997 - April 1999
4. TITLE AND SUBTITLE Toxicity of Experimental Jet Fuel System Ice-Inhibiting Agents: II. Gene Expression Response Profiling			5. FUNDING NUMBERS Contract F41624-96-C-9010 PE 62202F PR 2312 TA 2312A2 WU 2312A202	
6. AUTHOR(S) Geiss, K., Frazier, J.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) ManTech-Geo-Centers Joint Venture PO Box 31009 Dayton, OH 45437-0009			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Human Effectiveness Directorate Air Force Research Laboratory Wright-Patterson AFB, OH 45433-7400			10. SPONSORING/MONITORING AGENCY REPORT NUMBER AFRL-HE-WP-TR-2000-0109	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION AVAILABILITY STATEMENT Approved for public release; distribution is unlimited.			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) The potential toxicities of compounds to replace current US Air Force operational chemicals is of particular interest. A group of jet fuel system ice-inhibiting agent alternatives currently being evaluated for performance include derivations of 1,3-dioxolane-4-methanols and 1,3-dioxanes. The experiments presented here were performed in support of Air Force Research Laboratory's Materials Directorate research on FSII alternatives. Our initial approach to evaluating potential toxicity utilized primary rat hepatocyte cultures and gene profiling assays for assessing acute toxicity. The use of gene profiling assays, as presented here assist in developing patterns of toxicity and biological effects for chemicals or groups of chemicals. These profiles can then be used assist in the selection of appropriate operational chemicals. Here, the potential toxicities of a group of FSIIIs is assessed.				
14. SUBJECT TERMS ice-inhibiting			15. NUMBER OF PAGES 29	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT UNCLASSIFIED	18. SECURITY CLASSIFICATION OF THIS PAGE UNCLASSIFIED	19. SECURITY CLASSIFICATION OF ABSTRACT UNCLASSIFIED	20. LIMITATION OF ABSTRACT UL	

THIS PAGE INTENTIONALLY LEFT BLANK.

TABLE OF CONTENTS

<u>Section</u>	<u>Page</u>
Table of Contents.....	iii
List of Figures.....	iv
List of Tables.....	v
List of Abbreviations.....	v
Preface.....	vi
I. INTRODUCTION.....	1
II. MATERIALS AND METHODS.....	2
III. RESULTS.....	7
IV DISCUSSION AND CONCLUSIONS.....	21
V. REFERENCES.....	23

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
FIGURE 1 - Effect of FSII Dosing on MTT Reduction in Hepatocytes.....	8
FIGURE 2 - Results of CAT-TOX(L) Assay for M-1.....	10
FIGURE 3 - Results of CAT-TOX(L) Assay for M-2.....	11
FIGURE 4 - Results of CAT-TOX(L) Assay for M-3.....	12
FIGURE 5 - Results of CAT-TOX(L) Assay for M-22.....	13
FIGURE 6 - Results of CAT-TOX(L) Assay for M-26.....	14
FIGURE 7 - Results of CAT-TOX(L) Assay for M-27.....	15
FIGURE 8 - Results of CAT-TOX(L) Assay for M-DE.....	16
FIGURE 9 - Results of CAT-TOX(L) Assay for M-DP.....	17
FIGURE 10 - Results of CAT-TOX(L) Assay for M-EM.....	18
FIGURE 11 - Results of CAT-TOX(L) Assay for M-G.....	19

LIST OF TABLES

<u>Table</u>	<u>Page</u>
TABLE 1 - Test Chemicals.....	3
TABLE 2 - Summary Table of Gene Promoter induction.....	20

LIST OF ABBREVIATIONS

%	Percent, grams per 100ml
°C	Degrees Celsius
DMSO	Dimethyl sulfoxide
FSII	Fuel system ice-inhibitor
h	Hour
HepG2	Human hepatoma cell line "HepG2"
M	Molar, moles/liter
M-1	2,2-dimethyl-[1,3]-dioxolane-4-methanol
M-2	[1,3]-dioxolane-4-methanol
M-3	2-methyl-[1,3]-dioxolane-4-methanol
M-22	2-methyl-1,3-propanediol
M-26	2,2,5-trimethyl-[1,3]-dioxane
M-27	2,5-dimethyl-[1,3]-dioxane
M-DE	2-(2-methoxyethoxy)-ethanol
M-DP	Oxydipropanol
M-EM	2-Methoxyethanol
M-G	Glycerol
min	Minute
mL	Milliliter
mM	Millimolar, millimoles/liter
MPDIOL	2-methyl-1,3-propanediol
MTT	tetrazolium salt
nm	Nanometer, 10 ⁻⁹ meter
uL	Microliters, 10 ⁻⁶ liter
uM	Micromolar, 10 ⁻⁶ molar

PREFACE

This report is one of a series of interim technical reports describing the results of Task 2 of the Predictive Toxicology Program conducted at AFRL/HEST. The Predictive Toxicology Program is a collaborative effort that involves scientists from the Materials Directorate (AFRL/MLPJ, Dr. Ruth Pachter and Dr. Steve Trohalaki) and Human Effectiveness Directorate of the Air Force Research Laboratory, in addition to outside academic scientists (Dr. George Mushrush, George Mason University). Predictive Toxicology research (JON# 2312A202) is supported by the Air Force Office of Scientific Research (AFOSR), under the direction of Dr. Walt Kozumbo (AFOSR). This report describes experiments concerning the application of *in vitro* toxicology methods for the assessment of fuel system ice inhibitor toxicity performed at AFRL/HEST and at a commercial laboratory (Xenometrix, Inc., Boulder, CO) under Study Number WP-0498. The results from Xenometrix, Inc. are provided, as is, and without interpretation or guarantee by Xenometrix, Inc. Interpretation and summary is provided by the authors. Mr. John Schneider was the study coordinator at Xenometrix. The research described in this report began July 1997 and was completed in April 1999 under U.S. Air Force Contract No. F41624-96-C-9010 (ManTech/Geo-Centers Joint Venture). LtCol. Stephen R. Channel served as Contract Technical Monitor for the U.S. Air Force, Air Force Research Laboratory, Operational Toxicology Branch.

The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

I. INTRODUCTION

The US Air Force and other DoD agencies have pursued the replacement of current operational chemicals with alternatives that pose less potential toxic risk. One area of interest is in fuel system ice inhibitors (FSIIs) [1]. Alternatives to glycol ethers, such as diethylene glycol monomethyl ether (M-DE), are being investigated. In addition, new FSIIs are being synthesized through Research and Development activities of the Materials Laboratory. Three derivatives of 1,3-dioxolane-4-methanol (M-1, M-2, and M-3) and two derivatives of 1,3-dioxane (M-26 and M-27) are evaluated in this report. In addition, M-DE and a number of other chemicals are evaluated as reference compounds.

The development of fuel system ice inhibitors is an example of an integrated approach to designing new operational compounds that are potentially less toxic, while exhibiting acceptable performance characteristics [2]. This project involves the Operational Toxicology Branch, Human Effectiveness Directorate (AFRL/HEST), as well as scientists from the Materials Directorate (AFRL/MLPJ) of the Air Force Research Laboratory. When undertaking the selection of chemicals that are to be developed and pursued, current DoD acquisition strategies take into consideration the potential toxicities associated with human exposure to those chemicals of interest [3,4]. The utilization of various *in vitro* testing approaches is intended to assist our ability to address the question of potential chemical toxicity in a strategic and timely fashion.

In these experiments, an initial assessment of *in vitro* toxicity was performed in primary rat hepatocyte cultures. The chemicals were then tested for the potential to induce stress related genes *in vitro*. The CAT-TOX(L) assay performed in-house and at a commercial laboratory (Xenometrix, Boulder, CO) measures the effects of the interaction of potential toxicants with the regulatory elements of certain stress-inducible genes. The induction of these genes is determined by observing the expression of a specific reporter gene, modulated by the regulatory elements of particular stress genes.

The results of the stress gene assay give insight into response profiles of a group of chemicals and may provide insight into their toxic mechanisms. This approach has been utilized previously by AFRL/HEST for the assessment of the toxicity of ammonium dinitramide (ADN), an experimental rocket propellant [5]

II. MATERIALS AND METHODS

Chemicals

Dr. George Mushrush (George Mason University, Fairfax, VA) provided the test chemicals used in these studies. Chemicals were stored at room temperature until preparation of the experimental dosing solutions. Chemical names/synonyms, CAS registry numbers, molecular formulas, and structures shown in Table 1. The chemicals fall into four general groups: dioxolanes (M-1, M-2, M-3); dioxanes (M-26, M-27); alcohols (M-22, M-G); and glycol ethers (M-DE, M-DP, M-EM).

In Vitro Toxicity

Animals

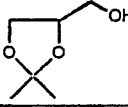
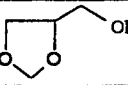
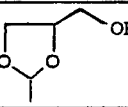
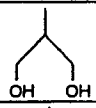
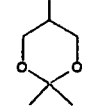
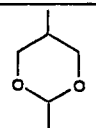
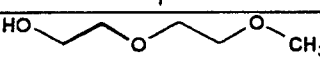
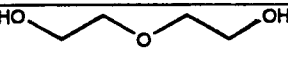


Male Fischer 344 rats (225-300 g, Charles River Breeding Laboratories) were anesthetized with 1 ml/kg of a mixture of ketamine (70 mg/ml; Parke-Davis, Moris Plains, NJ) and xylazine (6 mg/ml; Mobay Corp., Shawnee, KS) prior to undergoing *in situ* liver perfusion.

Liver Perfusion

Fischer 344 rat livers were perfused, and hepatocytes were isolated and enriched as previously described [6,7] with the following modifications. Perfusion medium (pH 7.2) was supplemented with 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Wash-out perfusion medium was supplemented with heparin (2.0 U/ml) and ethylenebis(oxyethylenenitrilo)-tetraacetic

acid (EGTA; 0.5 mM). Digestion perfusion medium was supplemented with collagenase (Roche/Boehringer Mannheim, Indianapolis, IN) at 0.26 U/ml (based on Wunsche U/mg).

TABLE 1. Test Chemicals

M-#	IUPAC Name (Synonyms)	Molecular Structure	CAS#	Molecular Formula
M-1	2,2-dimethyl-[1,3]- dioxolane-4-methanol (SOLKETAL)		100-79-8	C ₆ H ₁₂ O ₃
M-2	[1,3]-dioxolane-4-methanol (Glycerol formal)		5464-28-8	C ₄ H ₈ O ₃
M-3	2-methyl-[1,3]-dioxolane-4- methanol		3773-93-1	C ₅ H ₁₀ O ₃
M-22	2-methyl-1,3-propanediol (MPDIOL Glycol)		2163-42-0	C ₄ H ₁₀ O ₂
M-26	2,2,5-trimethyl-[1,3]- dioxane		25796-25-2	C ₇ H ₁₄ O ₂
M-27	2,5-dimethyl-[1,3]-dioxane		20615-12-7	C ₆ H ₁₂ O ₂
M-DE	2-(2-methoxyethoxy)- ethanol (Diethylene glycol mono- methyl ether; DIEGME)		111-77-3	C ₅ H ₁₂ O ₃
M-DP	Oxydipropanol (Dipropylene glycol)		25265-71-8	C ₆ H ₁₄ O ₃
M-EM	2-methoxyethanol (EGME)		109-86-4	C ₃ H ₈ O ₂
M-G	Glycerol		56-81-5	C ₃ H ₈ O ₃

Hepatocyte Enrichment and Culture

Primary rat hepatocytes were enriched by low speed (50xg) centrifugation. Typical viabilities of isolated hepatocytes ranged from 80 to 90%. For cell culture studies, primary hepatocyte suspensions were adjusted to a cell density of 1.0×10^6 cell/ml in DMEM culture medium (pH 7.2; Gibco, Grand Island, NY), containing gentamycin (50 mg/ml), 5% fetal calf serum (FBS; Gibco, Grand Island, NY). For dose-response studies, 1 ml aliquots of cell suspension (1×10^6 cells) were seeded into each well of Falcon (Becton Dickinson, Oxnard, CA) 6-well plates. After 3 h of incubation in a CO₂ incubator at 37°C to allow for attachment, rat hepatocytes were refed with DMEM culture medium and incubated at 37°C for 24 h prior to exposure.

Chemical Dosing

Stock dosing solutions were prepared fresh for each chemical at the start of a 48 h exposure. The chemicals were diluted in the appropriate medium as mentioned above. Existing media was removed before addition of dosing media (2ml/well for 6-well plates). Cells were incubated in dosing media for 48 h.

MTT Assay

The intracellular reduction of the tetrazolium salt (MTT) to blue formazan is an indicator of cell viability [8]. The MTT test represents a simple colorimetric method to determine cytotoxicity. The color change can be measured spectrophotometrically in a micro plate reader at 570 nm after incubation of the MTT substrate with cell cultures.

Induction of Stress Gene Expression

CAT-TOX(L) Assay

All experiments using the CAT-TOX(L) assay, performed either in-house or at Xenometrix (Boulder, CO), were carried out using the same test protocol [9]. Mammalian stress gene promoter constructs were synthesized by fusing individual promoter sequences for specific stress response genes with a reporter gene. The

activity of the reporter gene is measured by colorimetric assay. The reporter gene constructs have been stably transfected into the immortal human liver cell line, HepG2. Cells containing a single construct are exposed to a range of doses for each chemical. The response of the cells with a specific reporter reflect the influence of the chemical on that promoter. In these experiments, metabolic activation with S9 fraction was not utilized.

HepG2 cells, in 96-well plates were initially treated with four different dose levels (0 mM, 0.1 mM, 1 mM, and 10 mM) of chemicals M-1, M-22, or M-26. The commercial assays were performed with 0.0, 0.01, 0.1, 1.0, 10.0, 100.0 mM concentrations of test chemical (M-1, M-2, M-3, M-22, M-26, M-27, M-DE, M-DP, M-EM, M-G). M-26 and M-27 dosing solutions also contained 10% v/v of DMSO. Plates were incubated at 37°C, 5%CO₂, for 48 h. At the end of the exposure period, cells were washed and lysed. Following lysis, an aliquot of the supernatant was used to determine total protein content by colorimetric reaction and subsequent measurement at OD₆₀₀. Another aliquot was transferred to plates previously coated with anti-chloramphenicol acetyltransferase (anti-CAT) antibodies. Final detection was effected by incubation with a horseradish peroxidase conjugate, resulting in a color change that was measured at OD₄₀₅. Both the OD₆₀₀ and the OD₄₀₅ readings were used by the Xenometrix analysis software to determine the fold induction of stress genes.

Gene Construct Descriptions [From Ref. 9]

CYP1A1- The Cytochrome P450 1A1 (CYP1A1) enzyme is involved in the oxidative biotransformation of a number of xenobiotics. It is known to be induced in response to exposures to various chemicals, particularly polycyclic aromatic hydrocarbons (PAHs), which interact with the aryl hydrocarbon (Ah) receptor to regulate CYP1A1 expression.

CRE- The cAMP response element (CRE) is regulated in response to intracellular cAMP levels. Responses by this promoter reflect activation of cellular signaling pathways mediated by protein kinases.

FOS- *C-fos* (FOS) is an immediate early gene, which forms part of the AP-1 transcription activation complex. This promoter responds to DNA damaging factors, such as UV-irradiation or xenobiotic DNA-damaging chemicals, as well as resulting from heat shock or cellular oxidative stress conditions.

GADD153- GADD153 is the 153kDa growth arrest and DNA damage gene. Cell growth arrest and DNA damage trigger the activity of this CCAAT sequence-binding protein. UV-irradiation and exposure to chemicals, are noted inducers of this promoter.

GADD45- GADD45 is the 45kDa growth arrest and DNA damage gene. This promoter responds in a similar fashion as the GADD153 promoter. *In vivo*, the GADD45 gene contains a p53 response element. This promoter has been shown to respond to MNNG and calcium ionophores.

GSTYA- This is the promoter for the subunit Ya of glutathione-S-transferase (GST). GSTs catalyze the conjugation of a reduced glutathione molecule with a variety of electrophilic xenobiotics. This promoter responds to chemicals, such as PAHs.

HMTIIA- Metallothionein is a metal detoxification protein. Here the promoter for metallothionein-IIA (HMTIIA) is used. Along with heavy metals, glucocorticoids stimulate the induction of this heavy metal binding protein promoter.

HSP70- This is the promoter for the 70kDa heat shock protein (HSP70). Heat shock proteins belong to a family of protein chaperones that respond to high temperatures, and other physiological and chemical stressors.

XRE- The xenobiotic response element (XRE) promoter responds to exposures to different toxic chemicals. This sequence is part of the CYP1A1 promoter element. PAH's and polychlorinated biphenyls are known inducers of this promoter.

NFkBRE- NFkB is a gene responsible for rapid activation of genes involved in inflammatory, immune, and other acute phase responses. This promoter (NFkBRE) responds to cytokines, mitogens, and UV-damaged DNA.

p53RE- P53 plays a critical role in the modulation of the cell cycle and associated gene expression. The p53 response element (p53RE) responds to DNA damaging and growth arresting agents.

RARE- This is the retinoic acid response element (RARE). Retinoid compounds play primary roles in regulation of growth and differentiation. RARE responds to retinoic acid analogs.

GRP78- GRP78 is a 78kDa glucose-regulated protein. This molecular chaperone responds to DNA-damaging agents and intracellular calcium levels.

Data Analysis

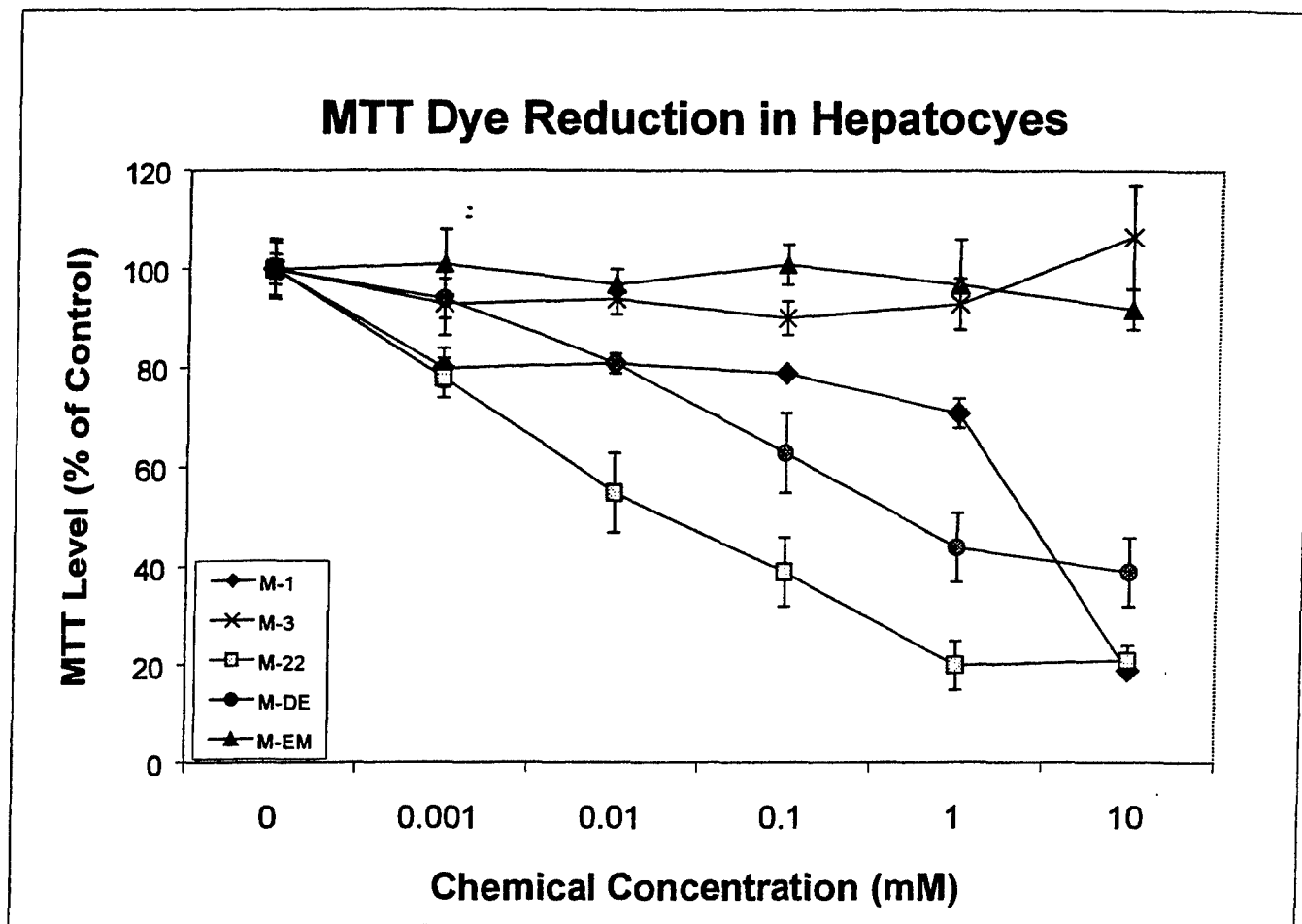
Statistical significance of CAT-TOX(L) induction was determined by ANOVA using the CAT-TOX analysis software with significance determined at $p < 0.05$.

III. RESULTS

In Vitro Cytotoxicity

Figure 1 shows the results of the MTT assay from the 48 h exposure of primary rat hepatocytes to the FSIIIs. The cells were dosed in triplicate. Figure 1 shows the mean and standard deviation of the three experiments. Further experiments are based on the response in this dose range for each chemical.

FIGURE 1. Effect of FSII Dosing on MTT Reduction in Hepatocytes after 48 hours



CAT-TOX(L) Assay

Preliminary Experiment

Three of the FSIIIs were tested (M-1, M-22, and M-26) with the CAT-TOX(L) kit to assess the general potential of these chemicals to elicit a response with this particular gene expression kit. Given the response, the decision would then be made whether to pursue the assessment of the full group of FSIIIs. The assay was performed once with each chemical. Since there was only a single experiment, these data were not subjected to statistical analysis. Cytotoxicity, as assessed by MTT assay, was not of

concern within the proposed dose range (0.1 - 10 μ M). Inductions of the gene promoters are considered relevant when they reach 2-fold or greater versus the expression in the control group. Ten of the 14 gene promoters responded to exposures within the dose range.

Definitive Experiment

Based on the preliminary experiment, the commercial gene profiling assessment of the ten FSIIIs was pursued. The dose range was expanded to include doses from 0.01 to 100 mM. Figures 2 - 11 show the results of the experiments. In order to present the data effectively, only the responses that were determined to be statistically significant ($p < 0.05$) are shown in the figures. Data are presented as the fold-induction as compared to control (no chemical treatment) group. The cell viability assay results are plotted on the same graph. The data are shown as the mean and standard deviation of three separate experiments. For the CAT-TOX(L) assay, gene promoter inductions in a range greater than 2-fold and statistical significance are the general criteria for determining whether the result is biologically relevant [9].

Table 2 shows a qualitative summary of those gene promoter responses that could be considered relevant (>2 -fold increase). For the FSII chemicals, the doses of 0.01, 0.1, and 1 mM did not result in gene promoter inductions that would be considered relevant. Therefore, only results from the 10 and 100mM dose groups are included in the table. Chemicals listed in three rows for each gene to emphasize the response by general groups of the chemicals (See Table 1). M-1, M-2, M-3 are dioxolanes. M-26 and M-27 are dioxanes. Linear molecules, alcohols M-22 and M-G and glycol ethers, M-DE, M-DP, and M-EM are listed on the third line.

FIGURE 2. Results of CAT-TOX(L) Assay for M-1

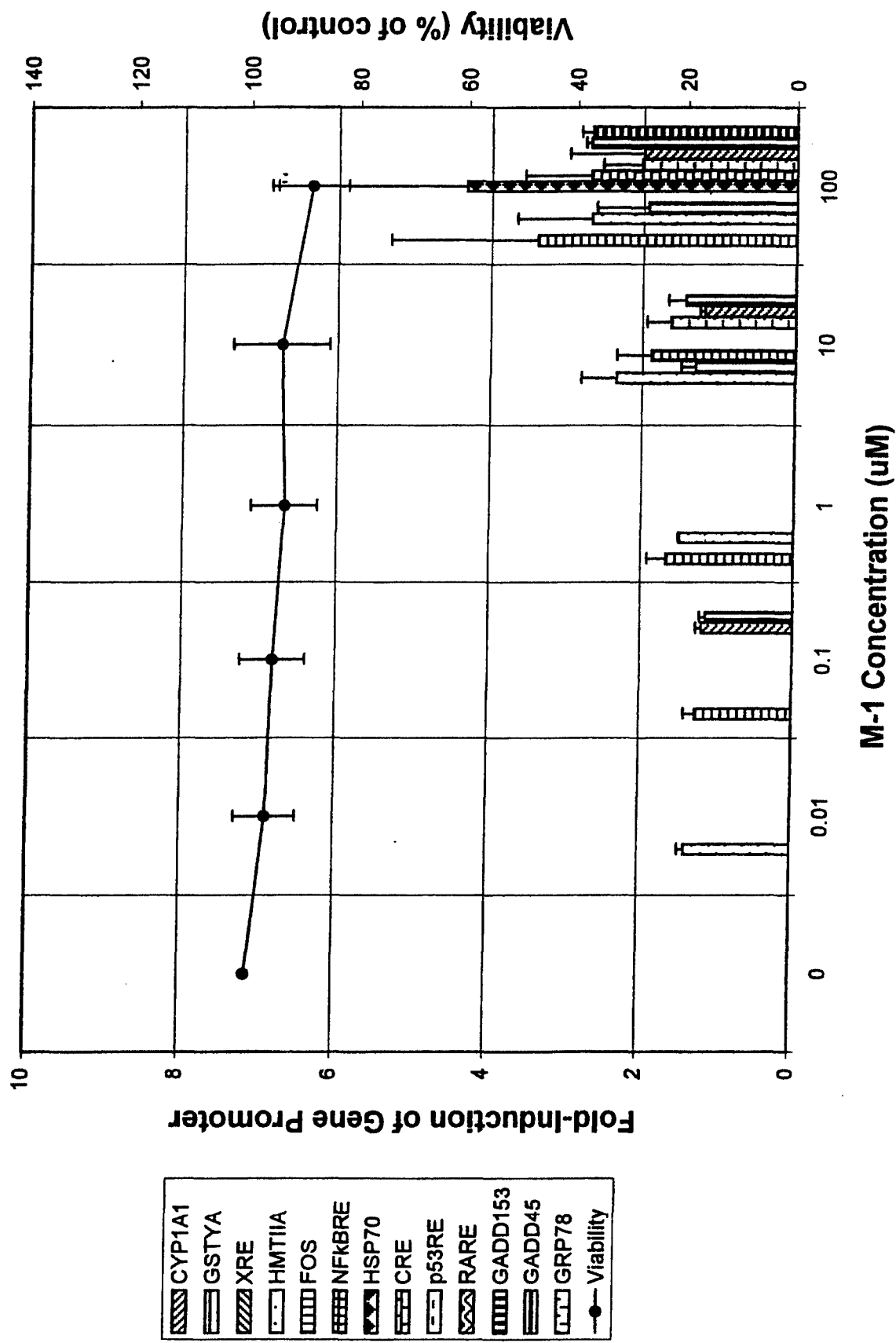


FIGURE 3. Results of CAT-TOX(L) Assay for M-2

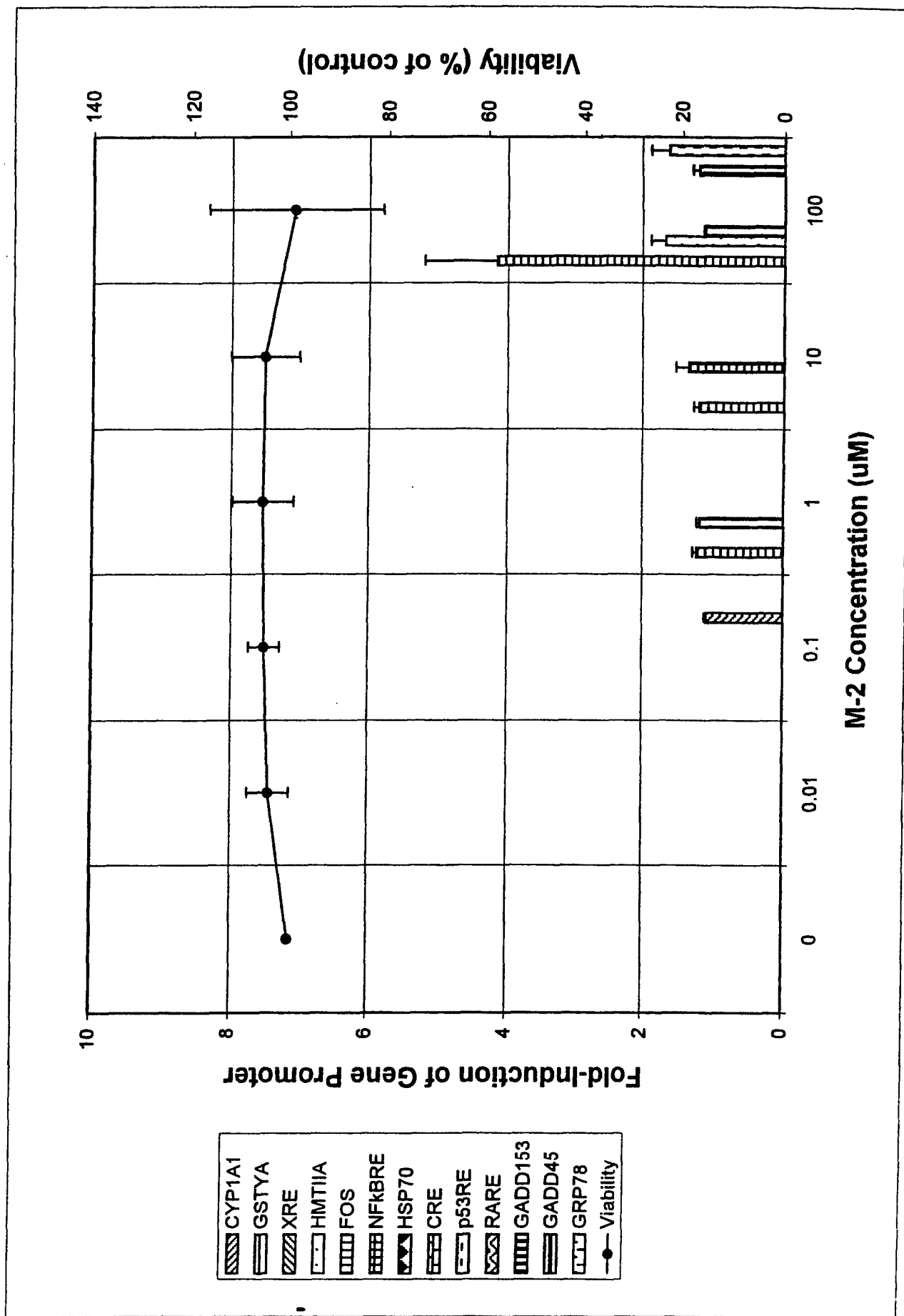


FIGURE 4. Results of CAT-TOX(L) Assay for M-3

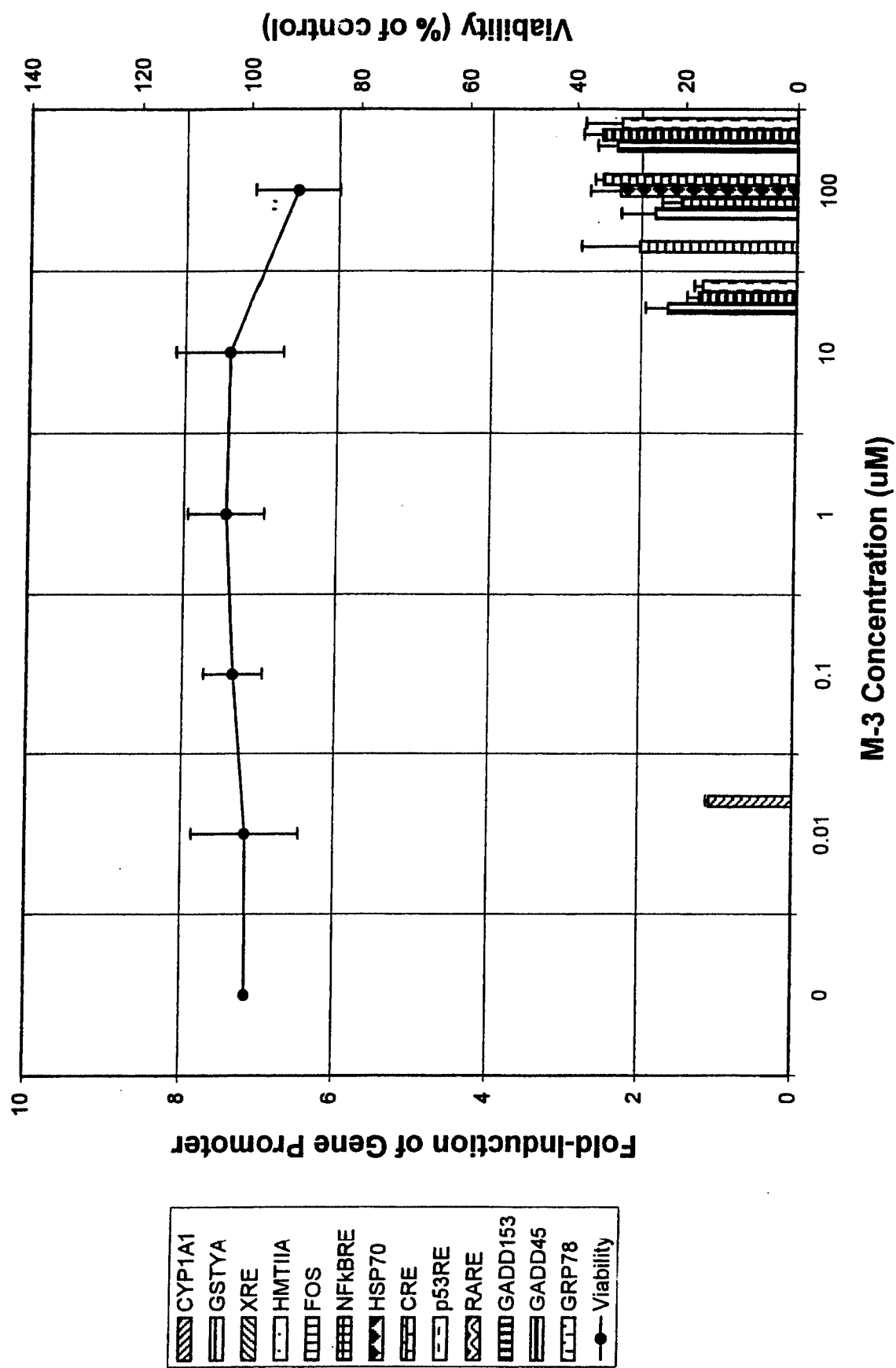


FIGURE 5. Results of CAT-TOX(L) Assay for M-22

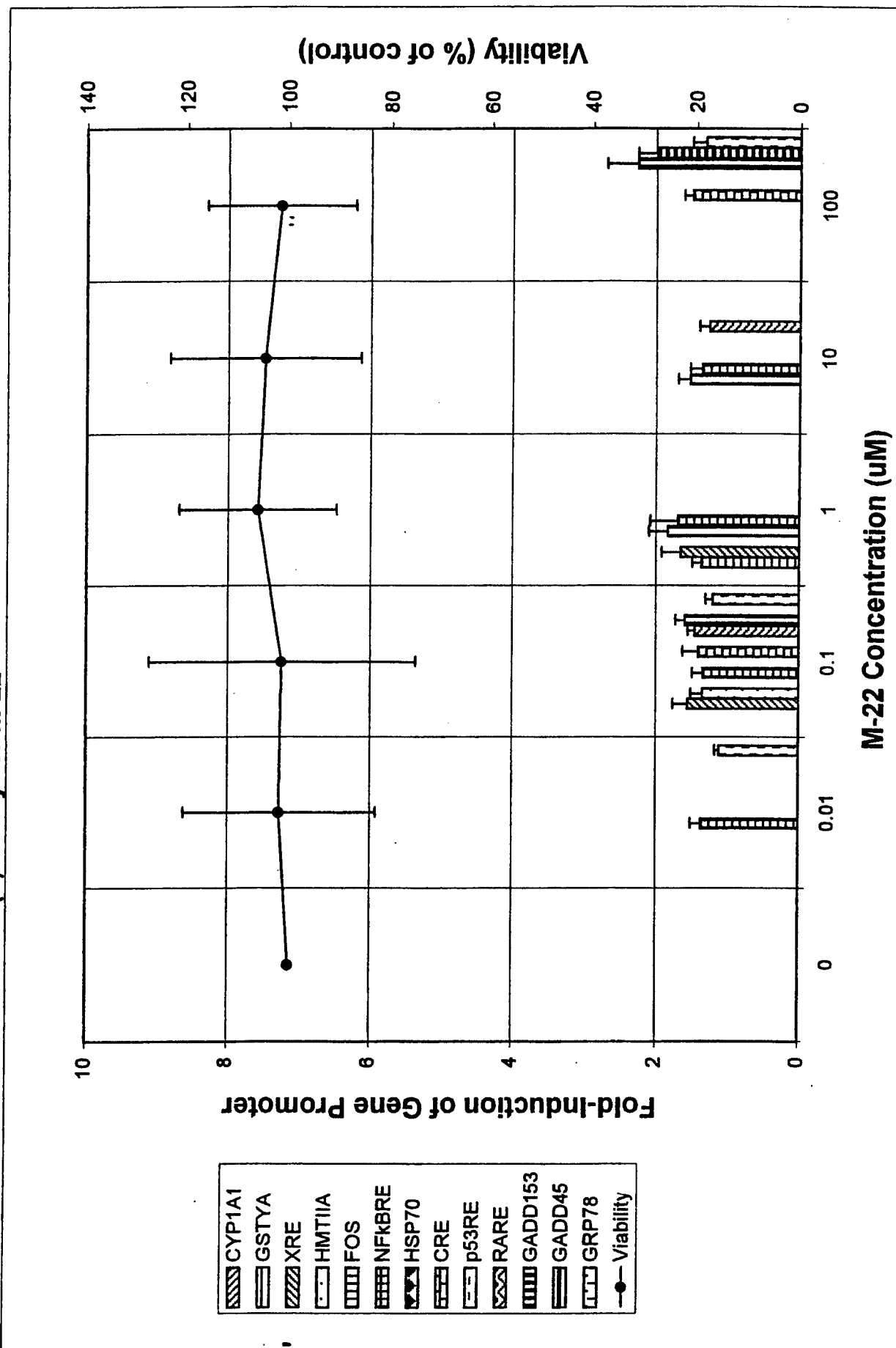


FIGURE 6. Results of CAT-TOX(L) Assay for M-26

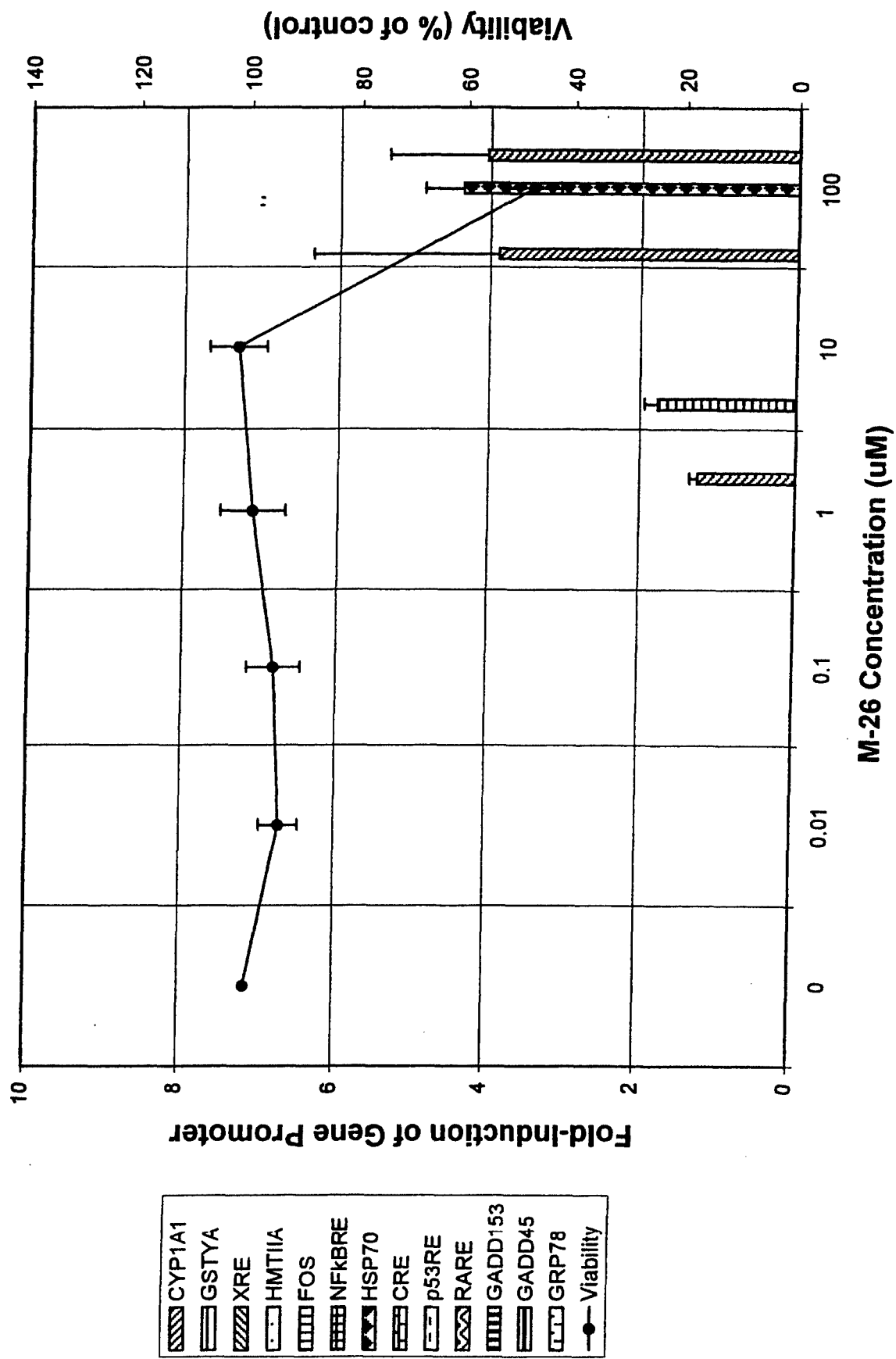


FIGURE 7. Results of CAT-TOX(L) Assay for M-27

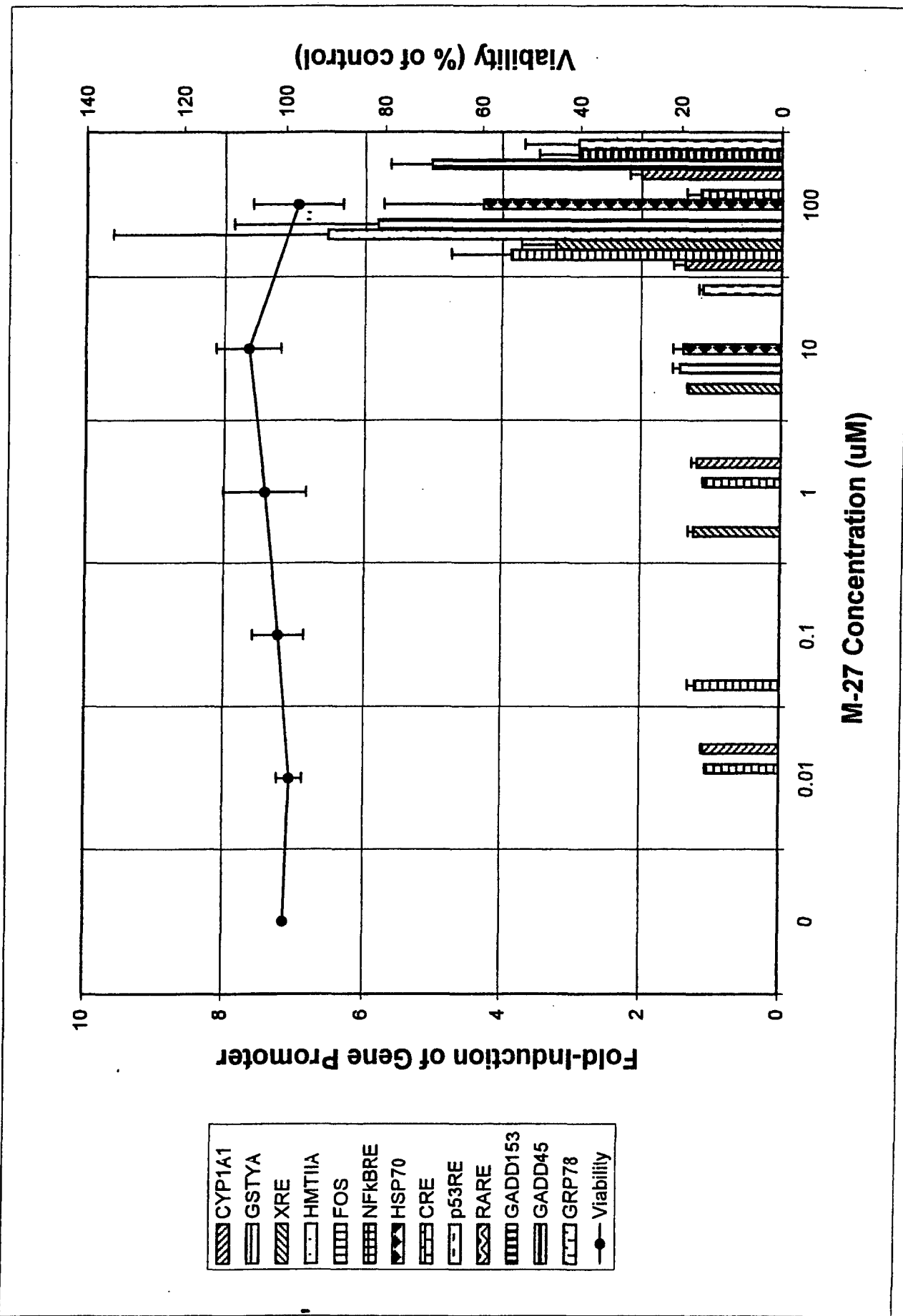


FIGURE 8. Results of CAT-TOX(L) Assay for M-DE

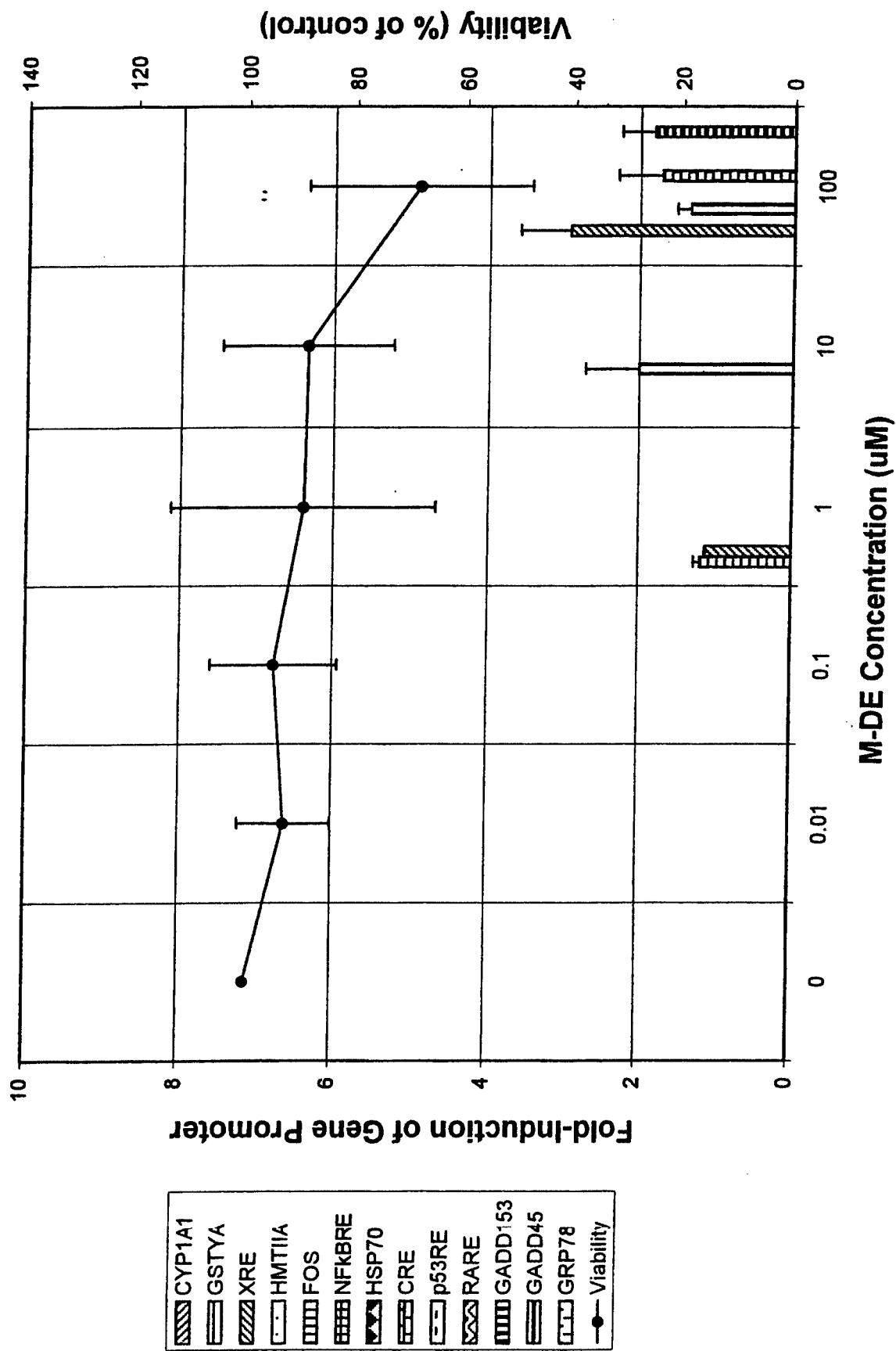


FIGURE 9. Results of CAT-TOX(L) Assay for M-DP

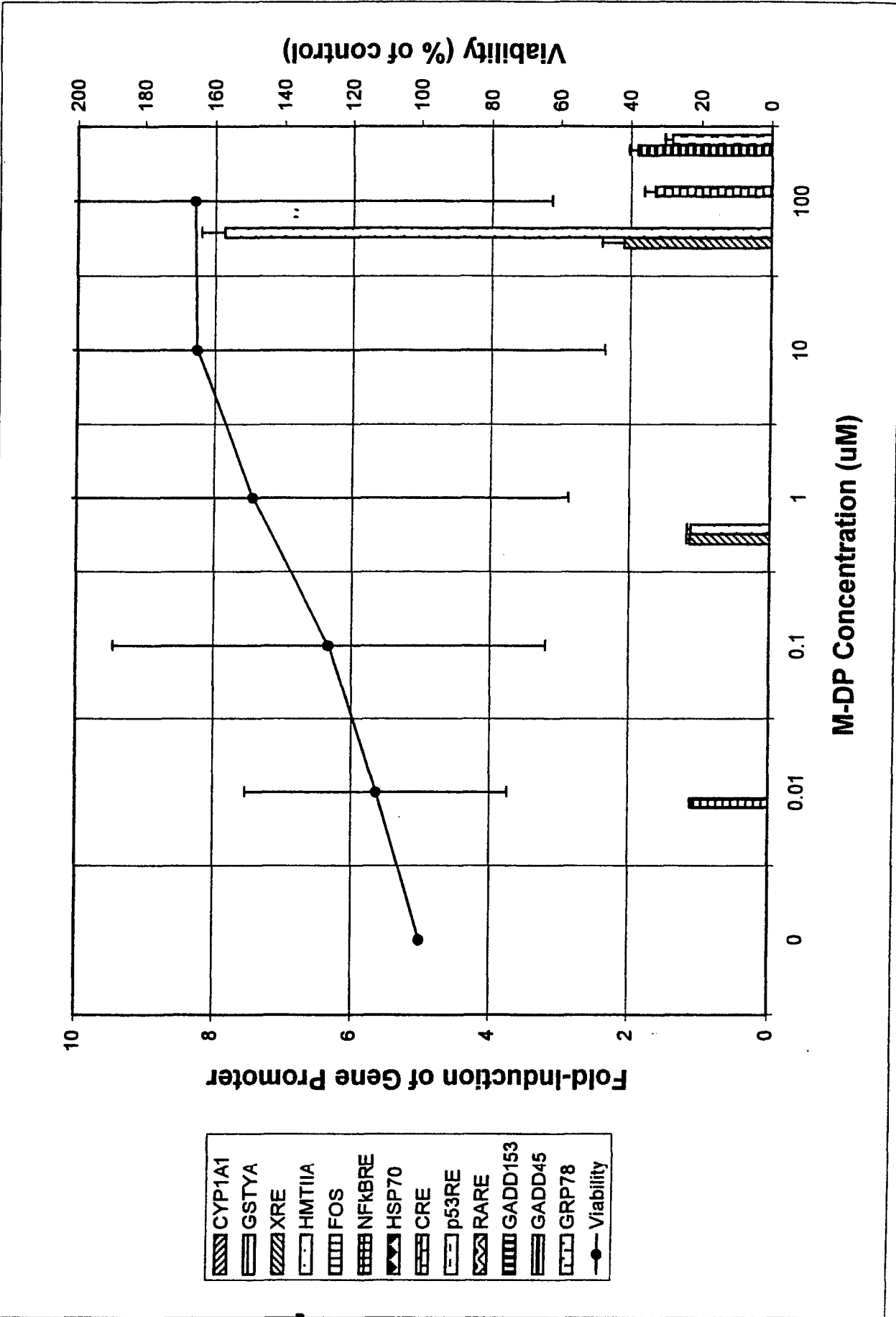


FIGURE 10. Results of CAT-TOX(L) Assay for M-EM

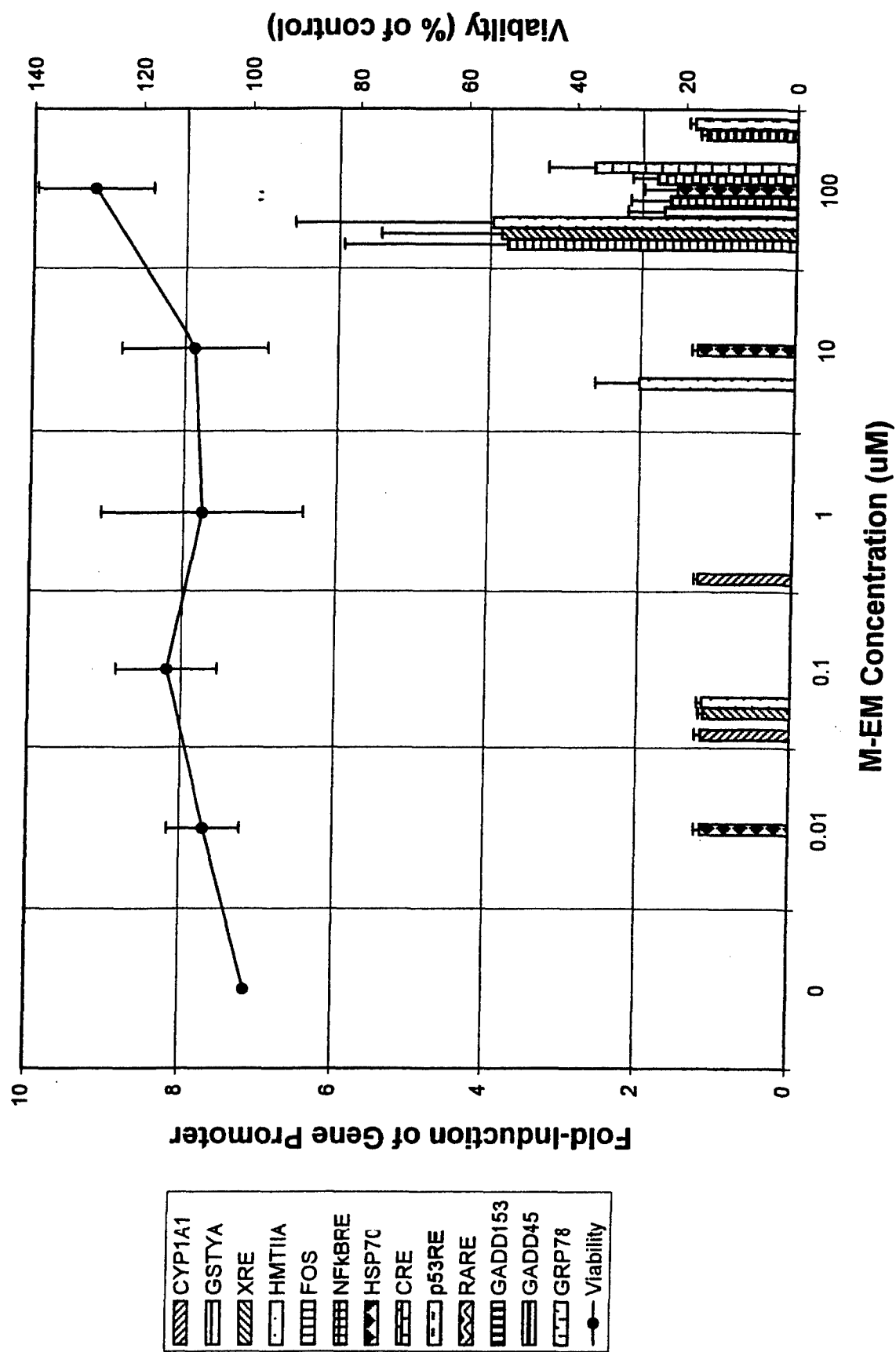


FIGURE 11. Results of CAT-TOX(L) Assay for M-G

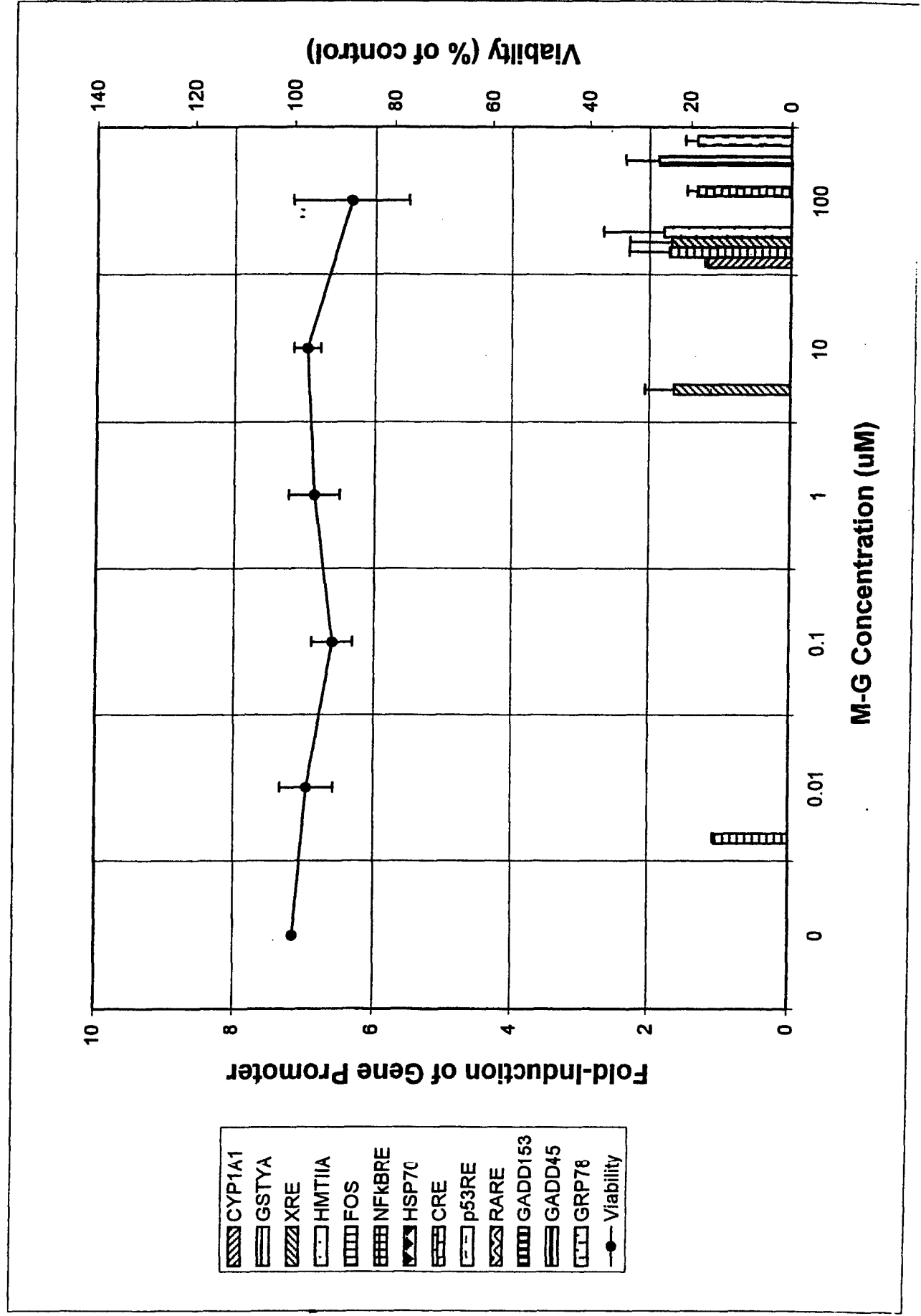


TABLE 2. Summary Table of Gene Promoter Induction

	10mM	100mM
CYP1A1	- - -	- M-26 -
GSTYa	- - -	M-1, M-2, M-3 M-27 M-EM
XRE	- - -	- M-27 M-DE, M-DP, M-EM
HMTIIA	M-1 M-EM -	M-1 M-27 M-DP, M-EM
FOS	- - M-DE	- M-27 -
NFkBRE	- - -	- - -
HSP70	- - -	M-1, M-3 M-26, M-27 -
CRE	- - -	M-1, M-3 - -
p53RE	- - -	M-1 - M-EM
RARE	- - -	M-1 M-26, M-27 -
GADD153	- - -	M-1, M-3 M-27 M-22
GADD45	- - -	M-1, M-3 M-27 M-22
GRP78	- - -	M-3 M-27 -

IV. DISCUSSION AND CONCLUSIONS

The cytotoxicity of the FSIIIs was scrutinized on both rat hepatocytes and HepG2 cells (as part of the CAT-TOX(L) assay). Of those tested, M-1, M-22, and M-DE displayed a dose-related decrease in MTT reduction. There tended to be lower cytotoxicity of specific doses FSIIIs in the HepG2 cells than what was seen in the rat hepatocytes after FSII dosing (Fig. 1 compared to Figs. 2-11). For some chemicals (M-1, M-22, M-DE) the viability measures for the HepG2 cells were markedly elevated at the same dose compared to that in the rat hepatocytes. M-1, M-22, and M-DE all displayed greater cytotoxicity to the rat hepatocytes, as measured by MTT, compared to the cytotoxicity observed for the same doses in the HepG2 cells. LC₅₀ calculations were not performed for the HepG2 cells, since the dose ranges were not high enough to observe full dose-responses for all chemicals. The average MTT response for M-DP seemed to increase, according to dose, up to 165% of control, yet the variation was very large. For M-EM, there was a significant increase in MTT response at 100 mM (128% of control).

A few initial comments can be made concerning the CAT-TOX(L) results. First, all promoters, exhibited a significant response for at least one chemical. However, none of the chemicals produced a greater than 2-fold increase in the NFkBRE response. Second, all chemicals produced a significant response (usually at the highest dose) with at least one gene promoter. Third, only three chemicals elicited a relevant response at the 10 mM dose (Table 2) with no responses for any promoters below that dose. Last, cytotoxicity was not a concern in the dose range used for this study except for M-26. As a result, the gene expression response for M-26 at 100mM is suspect. This cytotoxicity does not seem to correlate with what is observed for M-27.

Of the three dioxolanes (M-1, M-2, M-3), dosing with both M-1 and M-3 resulted in response for a number of the promoters. M-2 elicited a response from only the GSTYA promoter, which responded to the other dioxolanes, as well. A potential explanation for

this may be linked to the difference in the structures (Table 1). M-2 lacks any methyl groups on the 2-carbon. The other two dioxolanes (M-1 and M-3) are methylated at the 2-carbon position. Strictly by number of responding promoters, the response is proportionate to the number of methyl groups on the 2-carbon: M-1 (2 methyls, 8 induced promoters) > M-3 (1 methyl, 5 induced promoters) > M-2 (not methylated, one induced promoter).

A possible opposite effect is evident with the two dioxanes (M-26 and M-27). The effect of toxicity confounds the interpretation. Dosing with M-27 at 100 mM results in response from a larger number of promoters. M-26 has a much lower response at 100 mM, as measured by MTT. However, M-26 is more cytotoxic, as measured by the MTT assay. Although, not above the 2-fold threshold, M-27 does elicit responses from more in the gene promoters at doses less than 100 mM. In contrast to the dioxolanes, the chemical with more methyls at the 2-carbon (M-26) exhibited a response with fewer promoters (three).

Of particular interest to the Air Force would be how these potential FSII replacements compare to the current FSII, DiEGME (M-DE). M-DE dosing resulted in a relevant response for only two promoters (FOS and XRE). However, it was the most toxic at 10 mM, and the second most toxic of the chemicals tested at 100 mM, as measured by MTT in the HepG2 cells. On the other hand, in the gene reporter assay, only M-26 gave fewer statistically significant inductions in the gene reporters. Thus, it seems that M-DE was generally more cytotoxic, yet had less influence on stress gene expression than most all of the other FSII's. Our initial assessment of potential M-DE cytotoxicity by the rat hepatocyte experiments was consistent with the HepG2 results. This should be noted because for other chemicals (M-1 and M-22) the HepG2 cells exhibited less cytotoxicity as compared to the rat hepatocytes. M-2 exhibited less toxicity (by MTT in the HepG2 cells) and a similar low response in the gene reporter assay than M-DE.

V. REFERENCES

1. Mushrush, GW; Beal, EJ; Hardy, DR; Hughes, JM; and Cummings, JC. 1999. Jet fuel system icing inhibitors: Synthesis and characterization. *Industrial Engineering and Chemical Research* 38:2497-2502.
2. Geiss, KT; Bachowski, S; Frazier, JM; and Oris, JT. 1997. Development of a quantitative *in vitro* battery for the assessment of glycol ether toxicity. *Annual Meeting of the Society for Environmental Toxicology and Chemistry, San Francisco, CA*.
3. Geiss, KT; Channel, SR; and Frazier, JM. 1998. Comparative molecular toxicities of glycol-based ice-inhibiting agents. *The Toxicologist* 42:315.
4. Geiss, KT; Bachowski, S; and Frazier, JM. 1998. Potential role of oxidative stress in the *in vitro* toxicity of 1,3-dioxolane derivatives. *Free Radical Biology and Medicine* 25(Supp 1):S22.
5. Dean, KW and Channel, SR. 1995. *In Vitro* effects of ammonium dinitramide. Technical Report. *AL/OE-TR-1995-0059*.
6. Klaunig, JE; Goldblatt, PJ; Hinton, DE; Lipsky, MM; Chacko, J; and Trump, BF. 1981. *In Vitro*. 17:913-925.
7. Klaunig, JE and Baker, TK. 1994. In Tyson, CA and Frazier, JM (eds) *Methods In Toxicology: In Vitro Toxicity Indicators*, Academic Press, San Diego, vol. II, pp 72-80.
8. Al Casey, S; Brewster, D; Viau, C; and Acosta, D. 1995. Effect of glutathione depletion and oxidative stress on the *in vitro* cytotoxicity of velnacrine maleate. *Toxicology Letters* 76, 257-265.
9. Xenometrix, Inc. CAT-TOX(L) Assay Users Manual, v. 2.